Inhibition of LPS-stimulated NO Production in Mouse Macrophage-like Cells by Barbados Cherry, a Fruit of *Malpighia emarginata* DC

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Abstract. *The extract of Barbados cherry (acerola fruit), a fruit of Malpighia emarginata DC., has been reported to display diverse biological activities such as prevention of age-related diseases. We investigated here the possible effect of Barbados cherry extract on nitric oxide (NO) production by activated macrophages. Barbados cherry was roughly separated into 4 or 5 fractions by two different methods, using various organic solvents such as hexane, acetone, methanol (70% and 100%) and water, and assayed for its ability to inhibit NO production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells. Among these fractions, AcOEt extracts* (AE0) *in Method I and acetone extract* (A0) *in Method II showed the highest inhibitory activity of NO production (SI>20 and SI=31, respectively). When these fractions were subjected to silica gel column chromatography, higher inhibitory activity for NO production was concentrated in AcOEt* (AE6) *(SI=64) and benzene-AcOEt (1:4)*(A10) *fractions (SI>59). Western blot analysis demonstrated that* AE6 *and* A10 *fractions reduced the intracellular concentration of inducible NO synthase (iNOS) by approximately one-third. ESR spectroscopy showed that these fractions scavenged various radical species such as superoxide anion (O_2^-) and NO radicals. These data suggest that the inhibitory effect on NO production by Barbados cherry extracts is partly due to the inhibition of iNOS expression, and scavenging of O_2^- and NO radicals.*

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Malpighia emarginata DC. (Malpighiaceae) has been cultured in the tropics and subtropics and the fruit of this plant is called Barbados cherry or acerola fruit. The extract of Barbados cherry has been used as a phytoestrogen replacement in folklore medicines (1). The extract has also displayed diverse biological activities such as prevention of age-related diseases, cancer, hypertension, arteriosclerosis or myocardial infarction, possibly due to higher contents of antioxidants such as vitamin C, β-carotene and minerals such as calcium and phosphorus, dietary fibers and a small amount of vitamin B (2-5). Pretreatment of mice with Barbados cherry extracts inhibited the initiation of lung carcinogenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (6). Barbados cherry extract is popular in our daily life, since it is used as an additive in soft drinks and chewing gums. We have recently found that hexane extract of Barbados cherry showed both tumor-specific cytotoxic activity and multidrug resistance (MDR) reversal activity, a mechanism in which the radical-mediated oxidation reaction is not involved (Motohashi *et al.*, submitted). In addition to this direct antitumor action, Barbados cherry extract may also affect the function of various immunocompetent cells. We investigated here the effect of Barbados cherry extract on nitric oxide (NO) production and inducible NO synthase (iNOS) expression by lipopolysaccharide (LPS)-stimulated mouse macrophage-like Raw 264.7 cells.

Materials and Methods

Materials. Barbados cherry (acerola fruit, fruit of *Malpighia emarginata* DC.) from Petrolina Pernambuco state, Brazil, was supplied by Nichirei Corporation, Japan. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS,

USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); gallic acid (Tokyo Kasei Chem. Co. Tokyo, Japan); hypoxanthine (HX), xanthine oxidase (XOD), diethylenetriaminepentaacetic acid (DETAPAC), phenylmethylsulfonyl fluoride (PMSF) (Sigma Chem. Co., St. Louis, MO, USA); 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) (NO generator), superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan).

Extraction and fractionation of Barbados cherry extracts by Method I. Barbados cherry fruits (20 kg) were homogenized with acetone in a blender for 6 minutes at room temperature. The resulting mixture was filtered and the pulp was separated. The clear extract was concentrated under reduced pressure to remove the acetone and the residue was successively extracted with hexane, AcOEt and 1-butanol at room temperature. The solvent was concentrated *in vacuo* and the hexane extract **[HE0]** (3.4 g), AcOEt extract **[AE0]** (23.2 g) and butanol extract **[BU0]** (36.7 g) were obtained, respectively. The resulting water phase was concentrated *in vacuo* to give **[WA0]** (1639 g). In addition, the first separated pulp was extracted with MeOH and the solvent was concentrated *in vacuo* to give **[ME0]** (76.2 g).

The AcOEt extract **[AE0]** (20.1 g) was applied to silica gel column chromatography, which was then eluted with a benzene-AcOEt gradient. The benzene-AcOEt (9:1) fractions **[AE1]** (0.48 g), benzene-AcOEt (4:1) fraction **[AE2]** (2.14 g), benzene-AcOEt (3:2) fraction **[AE3]** (2.85 g), benzene-AcOEt (1:1) fraction **[AE4]** (2.30 g), benzene-AcOEt (1:4) fraction **[AE5]** (1.99 g), AcOEt fraction **[AE6]** (1.27 g), AcOEt-MeOH (1:1) fractions **[AE7]** (6.95 g) and MeOH fractions **[AE8]** (0.6 g) were eluted stepwise.

Extraction and fractionation of Barbados cherry extracts by Method II. Dried powders of Barbados cherry fruits (4.31 kg) were successively extracted with hexane, acetone, MeOH and 70% MeOH at room temperature. The solvent was concentrated *in vacuo* and the hexane extract **[H0]** (22.5 g), acetone extract **[A0]** (173.4 g), MeOH extract **[M0]** (1201.3 g) and 70% MeOH extract **[70M0]** (893.3 g) were obtained, respectively. The acetone extract **[A0]** (30 g) was applied to silica gel column chromatography, which was then eluted with a benzene-AcOEt gradient. The benzene fractions **[A1]** (0.82 g), **[A2]** (0.22 g) and **[A3]** (0.12 g), benzene-AcOEt (24:1) fraction **[A4]** (0.5g), benzene-AcOEt (9:1) fraction **[A5]** (0.12 g), benzene-AcOEt (4:1) fraction **[A6]** (0.16 g), benzene-AcOEt (3:2) fractions **[A7]** (0.53 g) and **[A8]** (1.98 g), benzene-AcOEt (1:1) fraction **[A9]** (2.12 g), benzene-AcOEt (1:4) fraction **[A10]** (3.84 g), AcOEt fraction **[A11]** (2.43 g), AcOEt-MeOH (1:1) fraction **[A12]** (13.96 g), and MeOH fraction **[A13]** (1.17 g) were eluted stepwise.

Cell culture. Mouse macrophage-like Raw 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere (7, 8).

Assay for cytotoxic activity. The cytotoxic activity of Barbados cherry extracts was determined by MTT method, and expressed as absorbance at 540 nm of the MTT-stained cells. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (7, 8).

Assay for NO concentration. Near confluent Raw 264.7 cells were incubated for 24 hours with each test sample in phenol red-free DMEM supplemented with 10% FBS and the NO production by Raw 264.7 cells was quantified by Greiss reagent (Molecular Probes Inc, Netherlands), using the standard curve of NO_2^-. To eliminate the interaction between sample and Greiss reagent, we also measured the NO concentration in the culture medium without the cells, and subtracted it from that with the cells (3). The concentration which inhibited the LPS-stimulated NO production by 50% (50% effective concentration: EC_{50}) was determined from the dose-response curve (7, 8). The efficacy of inhibition of NO production was estimated by the selectivity index SI, which was calculated by the following equation: $SI = CC_{50}/EC_{50}$

Western blotting. The cell pellets were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA and 2 mM PMSF) for 10 minutes in ice water, and then incubated for 50 minutes at 4°C with TAAB ROTATOR. The cell lysates were centrifuged at 16,000 xg for 20 minutes at 4°C to remove insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). Equal amounts of the protein from cell lysates (10 μg) were mixed with 2 x sodium dodecyl sulfate (SDS)-sample buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.01% bromphenol blue, 1.2% 2-β mercaptoethanol), boiled for 10 minutes, applied to the SDS-7% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in Tris-HCl buffered-saline plus 0.05% Tween 20 for 1.5 hours and incubated with anti-iNOS antibody (1:1,000)(Santa Cruz Biotechnology, Delaware, CA, USA) for 1.5 hours at room temperature or overnight at 4°C, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature. Immunoblots were detected by Western Lightning™ Chemiluminescence Reagent *Plus* (Perkin Elmer Life Sciences, Boston, MA, USA).

Radical scavenging activity. The radical intensity of Barbados cherry samples tested was determined at 25°C, using ESR spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency) (9, 10). Instrument settings; center field, 336.0±5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT: gain, 500, time constant, 0.1 s; scanning time, 2 minutes.

The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 50 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO. The microwave power and gain were changed to 5 mW and 250, respectively. When NOC-7 (NO generator) and carboxy-PTIO (spin trapping agent) were mixed, NO was oxidized to NO_2, and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. NO radical intensity was defined as the ratio of signal intensity of the first peak of carboxy-PTI to that of MnO (7-9).

Statistical analysis. For comparison of mean values between two groups, the Student's *t*-test was used.

Table I. *Inhibition of NO production by LPS-stimulated Raw 264.7 cells by Barbados cherry extracts and fractions, prepared by Method I.*

Extract and Fraction	Cytotoxic activity CC_{50} (μg/mL)		Inhibition of NO production EC_{50} (μg/mL)	
	LPS (+)	LPS (-)	LPS (+)	SI
HE0	317	414	68	4.7
AE0	>1000	>1000	49	>20
AE1	379	373	20	19
AE2	1000	>1000	33	>30
AE3	>1000	>1000	89	>11
AE4	>1000	>1000	115	>8.7
AE5	>1000	941	23	>43
AE6	892	908	14	64
AE7	>1000	>1000	69	>14
AE8	>1000	>1000	260	>3.8
BU0	>1000	>1000	58	>17
WA0	>1000	>1000	330	>3.0
ME0	>1000	>1000	156	>6.4

Near confluent Raw 264.7 cells were incubated for 24 hours with various concentrations of each Barbados cherry extract or fraction in the presence or absence of 100 ng/mL LPS in phenol red-free DMEM supplemented with 10% FBS, and then the viable cell number and extracellular concentration of NO were determined.

Table II. *Inhibition of NO production by LPS-stimulated Raw 264.7 cells by Barbados cherry extracts and fractions, prepared by Method II.*

Extract and Fraction	Cytotoxic activity CC_{50} (μg/mL)		Inhibition of NO production EC_{50} (μg/mL)	
	LPS (+)	LPS (-)	LPS (+)	SI
H0	>1000	>1000	262	3.8
A0	782	742	25	31
A1	>1000	>1000	277	>3.6
A2	590	571	98	6.0
A3	250	236	43	5.8
A4	171	179	13	13
A5	145	167	17	8.5
A6	134	144	15	8.9
A7	473	610	19	25
A8	924	938	25	37
A9	>1000	>1000	118	>8.5
A10	>1000	>1000	17	>59
A11	>1000	>1000	23	>43
A12	>1000	>1000	29	>34
A13	>1000	>1000	113	>8.8
M0	>1000	>1000	250	>4.0
70M0	>1000	>1000	200	>5.0

Near confluent Raw 264.7 cells were incubated for 24 hours with various concentrations of each Barbados cherry extract or fraction in the presence or absence of 100 ng/mL LPS in phenol red-free DMEM supplemented with 10% FBS, and then the viable cell number and extracellular concentration of NO were determined.

Results

Inhibition of LPS-stimulated NO production. Barbados cherry was roughly separated into 4 or 5 crude extracts by two different methods, using various organic solvents such as hexane, acetone, methanol and water, and assayed for its activity to inhibit the NO production by LPS-stimulated mouse macrophage-like Raw 264.7 cells. Among these fractions, AcOEt extracts (**AE0**) (SI>20) in Method I and acetone extract (**A0**) (SI=31) in Method II inhibited the NO production, more potently than did other fractions [hexane extract (**HE0**) (SI=4.7), 1-butanol extract (**BU0**) (SI>17), water extract (**WA0**) (SI>3.0), MeOH extract (**ME**) (SI>6.4) in method I; hexane extract (**H0**) (SI>3.8), MeOH extract (**M0**) (SI>4.0), 70% MeOH extract (**70M0**) (SI>5.0) in Method II] (Tables I, II). When these extracts were subjected to silica gel column chromatography, higher inhibitory activity was concentrated in AcOEt (**AE6**) (SI=64) and benzene-AcOEt (1:4) fractions (**A10**) (SI>59), respectively (Figure 1, Tables I, II). This indicates that the specific activity of inhibitory activity of NO production was significantly increased during column chromatography. Both **AE6** and **A10** alone (without LPS) did not significantly induce NO production, but completely eliminated the LPS-stimulated NO production, even at non-cytotoxic concentrations (Figure 1).

iNOS expression. Western blot analysis demonstrated both **AE6** and **A10** fractions slightly, but not significantly ($p<0.26$ for **AE6;** $p<0.24$ for **A10** at 100 μg/mL) reduced the intracellular concentration of iNOS protein (by approximately one-third) (Figure 2).

Radical scavenging activity. ESR spectroscopy showed that **AE6** and **A10** produced only a trace amount of radical, and the radical intensity of these fractions was one-tenth that of gallic acid (Motohashi *et. al.*, submitted). On the other hand, **AE6** and **A10** effectively scavenged the O_2^- generated by HX-XOD reaction (115 and 14.8 SOD unit/mg, respectively), although the activity was two or three orders lower than that of gallic acid (22425 SOD unit/mg)(10) (Table III). These fractions also scavenged the NO produced by NO generator (NOC-7) in the presence of carboxy-PTIO (Table III).

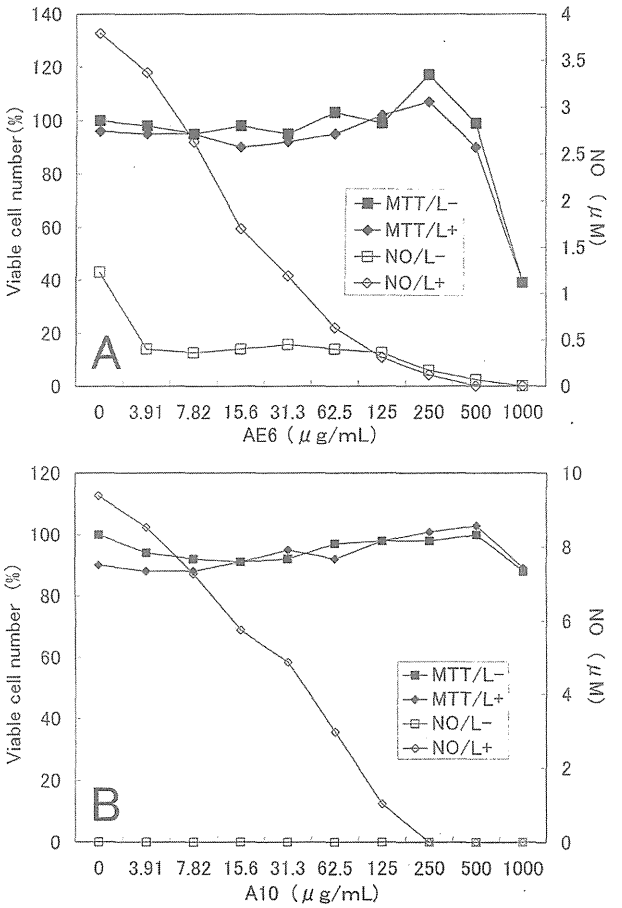

Figure 1. *Barbados cherry extracts inhibited the NO production by LPS-stimulated Raw 264.7 cells. Near confluent Raw 264.7 cells were incubated for 24 hours with the indicated concentrations of* AE6 *(A) or A10 (B) in the presence (◆, ◇) or absence (■, □) of 100 ng/mL LPS in phenol red-free DMEM supplemented with 10% FBS, and then the viable cell number (◆, ■) and extracellular concentration of NO (◇, □) were determined. Each value represents mean from 4 assays.*

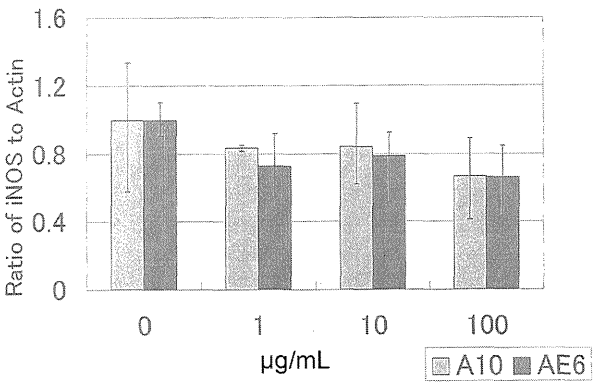

Figure 2. *Effect of Barbados cherry extracts on the intracellular concentration of iNOS protein in Raw 264.7 cells. The intracellular concentration of iNOS was expressed as the ratio to that of actin. Each value represents the mean±S.D. from three independent experiments.*

Table III. *Radical generation and scavenging activity of Barbados cherry fractions.*

Extracts and Fractions	Radical intensity[a] pH 10.5	12.5	Radical scavenging activity O_2^- (SOD unit/mg)	NO (EC_{50}: μg/mL)
AE6	0.08[b]	0.08[b]	115.0[b]	5.9
A10	0.14[b]	0.11[b]	14.8[b]	36.3
Gallic acid	1.30[c]	7.20[c]	22425.0[b]	3.8

[a]Sample concentration: 2 mg/mL except for gallic acid (3 mM).
[b]Motohashi *et al.*, submitted
[c]From ref. 10

Discussion

NO is produced from L-arginine by NOS in the presence of NADPH and displays diverse biological activities such as vasodilation, inhibition of endothelial leukocyte adhesion and regulation of energy metabolism (11). We have previously reported that LPS stimulated this cell line to produce tumor necrosis factor (TNF), NO, citrulline and asparagine (12, 13). We found that addition of Barbados cherry extract to LPS-activated macrophages reduced the extracellular concentration of NO to an undetectable level. This was partially due to the decreased expression of iNOS protein and radical scavenging activity of these extracts. We found that **AE6** and **A10** directly reduced the radical intensity of carboxy-PTI (Satoh *et al.*, unpublished data), raising the possibility that the NO radical scavenging activity of these extracts may be partly due to their reducing activity. This is supported by the finding that ascorbate radical was detected in solution of **AE-6** (data not shown).

The extracellular NO concentration may decline by the instantaneous capturing by thiol compounds or serum proteins to form *S*-nitroso adduct (14). However, in the presence of reducing agents such as ascorbic acid (15, 16) or copper (17), the nitrosothiol will be decomposed so as to release again NO. Another scavenging substance for NO is dioxygen, which significantly reduces the biological activity of NO (18). NO reacts with superoxide to form peroxynitrite, which has potent oxidizing activity (19). It is unclear whether peroxynitrite is produced at concentrations sufficient to damage the tissues *in vivo*.

We found that Barbados cherry extracts significantly reduced the extracellular NO concentration by whatever mechanism and are therefore expected to modify the biological action of NO radical. We found that the inhibitory effect of **AE6** and **A10** was greater than that of epigallocatechin gallate, gallic acid or phenylpropenoids (20). Further purification of fractions **AE6** and **A10** is necessary to identify the active principles.

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